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13. ABSTRACT (Maximum 200 Words)

The hypothesis of these studies is that the genomic processes that lead to the variations in both structural and material properties of bone development will be recapitulated in the developmental mechanism(s) that controls the bone's structural geometry and material properties during fracture healing. Two goals were set out in the proposal to test this hypothesis. The first was to determine how variations in basic bone structure and material properties in three in bred strains of mice is translated into the healing process of fracture repair in terms of callus structure and biomechanical properties. Towards this goal microCT analysis of day 21 and 35 fracture calluses have been carried out and each strain showed unique variations in geometric structure. The second goal of these studies was directed at identifying the underlying genomic processes that are activated and accompany fracture healing in the three genetic strains of mice through the use of full transcriptional profiling of gene expression over the fracture healing period. Towards completion of this goal an initial transcriptional profiling study in one of three strains B6 has been completed which both establishes our basic methodological approach to these studies and identifies several unique families of genes that are activated during fracture healing.

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INTRODUCTION

Studies have shown that individuals with a small moment of inertia (slender bones) are at higher risk of developing stress fractures during basic training (Beck et al., 1996; Milgrom et al., 1989). Because the size, shape and material properties of adult bones are determined early in life, it will be important to understand the biological processes (or factors) that contribute to variation in these morphometric and material traits in the context of the development of stress fractures. Understanding how the genetic background affects fracture healing will lead to further insights into skeletal tissue response to pharmacological agents that might promote fracture healing and predict if different individuals will heal at differing rates. The hypothesis of these studies is that the genomic processes that lead to the variations in both structural and material properties of bone development will be recapitulated in the developmental mechanism(s) that controls the bone's structural geometry and material properties during fracture healing. To test this hypothesis, simple transverse fractures will be generated in the femora of three strains of mice, A/J, B6, and C3H. These species have known differences in peak bone mass that have been shown to reside in both structural and material variations intrinsic to these genetic strains of mice. The ultimate goal of this proposal is to identify relational characteristics between the genetic components that regulate the variations in development of bone mass and geometry by comparison of fracture repair processes in the three strains of mice. Two goals were proposed to address the research hypothesis. The objective of goal 1 is to: a) Determine the nature of the quantifiable differences that are seen in the regeneration of the biomechanical and microarchitectural properties of fracture calluses over a 42 day healing period in the three strains of mice. b) Determine how the genetic strain variations in the microarchitectural properties relates to the rates of biomechanical healing as well as the relationship between micro architecture and regain of biomechanical strength. The objective of goal 2 is to: a) Determine the underlying genomic processes that are activated and accompany fracture healing in the three genetic strains of mice and identify strain variation in the genomic patterns of expression by carrying out both select and full transcriptional profiling of gene expression over the same healing period used in aims one b) Identify tissue based differences in the mechanisms and associated genes that are responsible for genetic strain variations in bone development and fracture healing.

BODY

<u>Progress towards completion of Goal 1</u>: Two of three post fracture groups of mice (3 strains x 2 time points) have been completely enrolled for mechanical testing and micro CT. These included days 21 and 35. We revised the end point from 42 days to 35 days, since histological assessments of the 42-day post fracture calluses showed that most of the remodeling in the mouse calluses had been completed and it was decided to initially obtain mechanical and CT properties that would be more reflective of the period of primary bone remodeling and regain in mechanical property versus terminal rates of regain of strength. Micro CT was carried out on between 15- 20 calluses per strain per time point and representative micro CT images of the three strains at the two time points are presented in the figures with the graphical analysis of various whole bone properties that are reflective of the calluses at the two time points.

Fractured femurs were imaged using microComputed Tomography at a voxel resolution of ~7 microns (Figure 1). The microCT images clearly showed that at 21 days post-fracture, there was significant variation in the size and shape of the callus among the three inbred mouse strains. The average total bone area and the polar moment of inertia were quantified from the microCT images for the intact bones and the day 21 and day 35 fracture calluses for A/J, B6, and C3H inbred mice. Significant differences in the size of the callus were observed among the inbred mouse strains at 21 days (Figure 2). For A/J mice, the area and polar moment of inertia of the fracture callus were 321% and 1022% greater compared to the intact bone. In contrast, for B6 mice, the area and polar moment of inertia of the fracture callus were 525% and 2603% greater compared to the intact bone. For C3H mice, the area and polar moment of inertia of the fracture callus were 484% and 2286% greater compared to the intact bone. Thus, we showed

that B6 mice heal by creating a rather large callus, C3H an intermediate size callus, and A/J a very small callus. Thus, we showed that genetic background plays an important role in the fracture healing process.

By 35 days of age, the fracture calluses of A/J and B6 (C3H data is underway) showed considerable remodeling back to the size of the intact bone (Figure 2).

Preliminary mechanical testing was conducted by loading intact and fractured femurs to failure in torsion. Testing is underway for each of these experimental groups although we may have to generate additional fractures to reach statistical significance since a number of the femurs were dissected such that insufficient bone was left above the callus to appropriately pot them for torsion mechanical testing. To date, mechanical testing has been conducted on A/J femurs (n=5-6) and only a small number of B6 femurs. A/J femurs (intact and at day 21 and 35 of fracture healing) were evaluated for mechanical performance (Figure 3). Day 21 callus showed similar stiffness values as the intact bones, however, the torque-to-failure was reduced. By day 35, the stiffness was significantly greater than the intact bone and the torque-to-failure was equivalent to that of the intact. This data indicated that A/J femurs regained strength following fracture by 35 days of age. Statistical analyses will be conducted on this data once a sufficient number of samples have been tested. Preliminary mechanical testing of the B6 femurs showed that at day 35, the B6 femurs showed similar stiffness (4.2 $\pm$ 2.1 Nmm/deg) and torque-to-failure (33.2 $\pm$ 5.7 Nmm) compared to A/J. This was interesting because these two mouse strains showed very different fracture callus sizes at both 21 and 35 days of healing. This suggested that these inbred mouse strains are creating mechanically stable fractures in very different ways and that these different pathways may be mechanically equivalent.

Progress towards completion of Goal 2: Initial experiments have been completed that have allowed us to establish all of the experimental techniques and appropriate reference to normalize the expression data to carry out large scale transcriptional profiling across mouse fracture healing. One of the most important aspects of our first studies was the demonstration that pooled mRNAs from 12.5 pc and 17 pc day whole mouse embryos were inappropriate as the reference for these studies since many of the mRNAs that we had anticipated to be induced during endochondral phase of fracture repair were under-expressed relative to the reference. While we had initially proposed to use the pooled RNAs from embryonic bones as the reference to compare the fracture callus mRNA profiles, we found that this reference biased our results since many of these mRNAs were expressed during embryogenic bone development at many, many fold higher levels than in post natal repair. By comparison of our first series of studies we were able to determine that unfractured bone appeared to provide a more adequate base line to assess new gene expression during fracture healing. Using these initial trials and a limited set of time points, we were able to carry out a complete study that has resulted in a work that has been presented in three meeting and in the preparation of a manuscript for presentation. These results are summarized below and appended. During this same period we completed the enrollment and harvesting of fracture calluses and preparation of all of the mRNA samples for the detailed transcriptional comparison, in the three genetic strains.

Key Research Accomplishments

Accomplishments Goal 1: Standard mid-diaphyseal femur fractures were generated in A/J, C57BL/6J, and C3H/HeJ inbred mouse strains. The calluses at day 21 and 35 of healing were examined for morphological (via microCT) and mechanical (via torsion testing) differences. Significant variation in the size of the callus was observed among the inbred mouse strains that correlated well with the variation in external size of the intact bone. Intact B6 femurs show the greatest external diameter and also show the largest fracture callus. In contrast, intact A/J femurs show the smallest external diameter and also the smallest fracture callus. C3H femurs show intermediate values for both intact and fracture callus measures. These data indicated that genetic background plays an important role in the fracture healing

process. Each inbred strain achieved a mechanically stable fracture, but they achieved this in significantly different ways.

Accomplishments Goal 2: Standard mid-diaphyseal tibia fractures were generated in C57B6 murine tibiae and the transcriptional expression of ~13,000 genes was assessed using 70 nt oligo-nucleotide microarray slides. Using unfractured bone as the reference the transcriptional activity of genes with 2 fold greater or lesser levels of expression relative to the reference was identified. Using a self- organizing mapping approach, the temporal relationships between extracellular matrix protein expression and that of the proteases that degrade proteoglycan and collagenous matrices was assessed. A very broad group of extracellular matrix mRNAs representative of connective tissues in general and more specialized proteins expressed predominantly in basement membranes, blood vessels and in cartilage all showed an elevated pattern of expression over a 21 day period after fracture and their expression peaked at 10 days when the endochondral period of the bone formation was maximal. Specific metalloproteinase mRNAs were consecutively expressed with MMP 14 and 2 preceding MMP9 and MMP13 while the expression of MMPs 8 and 19 showed a strong inhibition of their expression during the first 21 days of bone healing. The metalloproteinase inhibitors (TIMPs 2 and 3 had an identical pattern of expression as MMPs2 and 14 while TIMP1 was induced early after fracture and remained highly expressed thereafter. The disintegrins/ADAMTS family of proteases also showed very unique patterns of expression with ADAMTS 4 and 15 paralleling the expression of MMPs2 and 14 and ADAMTS 1 following the expression pattern of MMPs 9 and 13. A second group (ADAMTS 17,19,5 and 8) showed a biphasic pattern of expression first being induced right after fracture then showing reduced expression during the endochondral period followed by a period of increasing expression during primary bone remodeling. These results demonstrate that an orderly temporal expression of MMPs and ADAMTs is necessary for the progression of endochondral resorption. Furthermore based on their patterns of expression, relative to the known activities of the encoded proteolytic enzymes, our results suggest that the dissolution of cartilage protoeglycans proceeds before the underlying collagenous components of the matrix are removed.

While not presented in the appended manuscript our analysis also identified the heat shock (HSP) gene family to be tightly expressed immediately after injury at day 3 and is the first demonstration that this family of stress related genes is activated in response to bone fracture. We are very excited about this observation since in the studies of soft tissue trauma the HSP have prognostic value to predict both extent and outcome for numerous kinds of injury. This family of genes is rapidly synthesized in response to many different types of environmental stresses including temperature changes, inflammation, irradiation, oxidizing agents, heavy metal ions, and anoxia (Kim, 2003). HSPs also carry out carry out wide variety of functional roles in response to injury (Prohaszka and Fust, 2004). They appear to participate the regulation of pro-inflammatory cytokines are molecular chaperones that recognize and form complexes with incorrectly folded or denatured proteins, and can regulate cellular apoptosis (Reynolds and Allen, 2003; Yamashita-Goto et al., 2002; Tsan and Gao, 2004). Their role as potent activators in the immune system in the absence of exogenous infective agents (Tsan and Gao, 2004) may partly explain their elevated levels of expression during the inflammatory phase of fracture repair. Finally, heat shock proteins have been observed in the serums from patients suffering from cardiovascular ischemia, burn, spinal injury, head trauma, anoxia and a number of inflammatory diseases and they may have prognostic value to patient outcome for these injuries as well as acute trauma (Kim, 2003; Pittet et al., 2002).
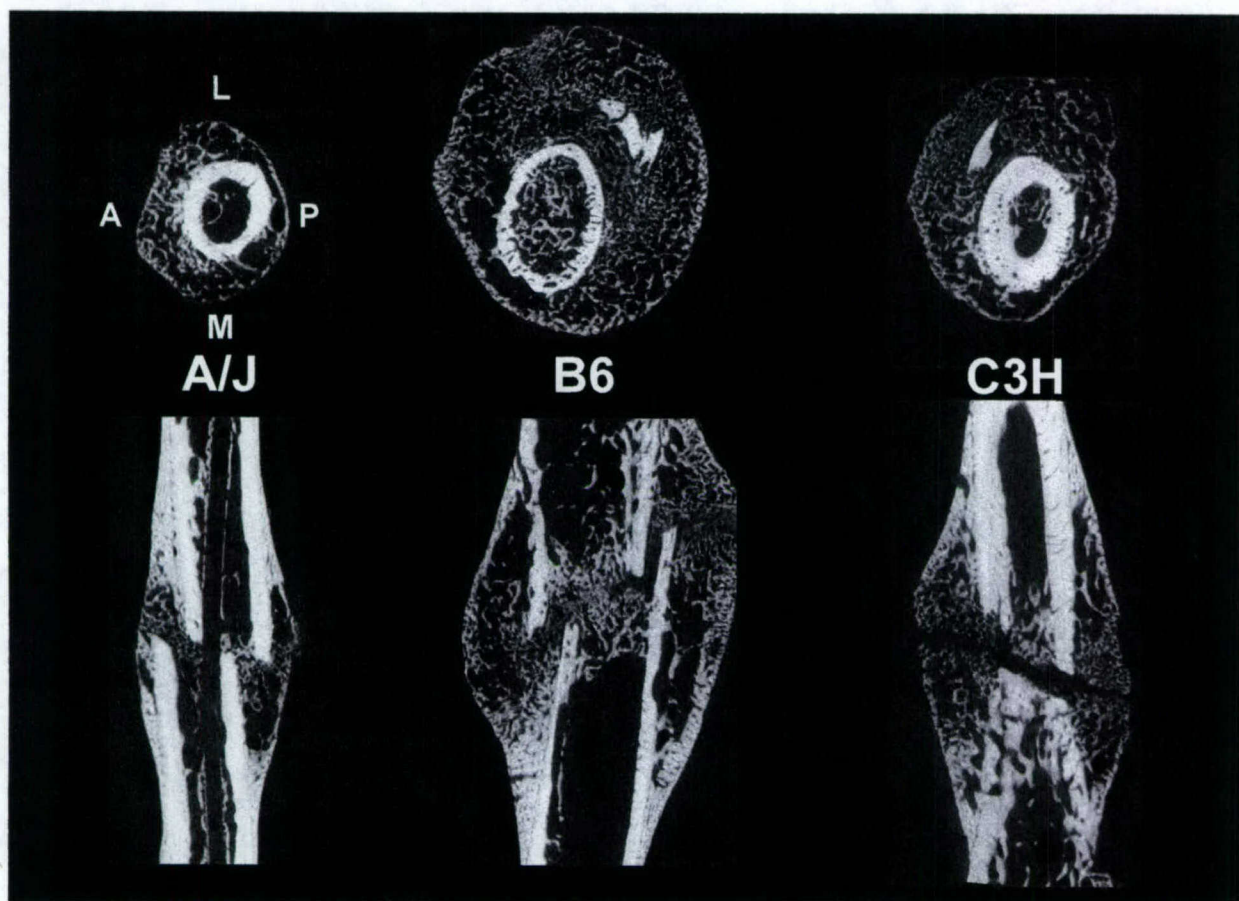
Figure 1. Transverse (upper) and sagittal (lower) images obtained using microComputed Tomography showing the variation in the size and shape of 21 day fracture calluses for three inbred mouse strains (A/J, B6, and C3H). All images are shown at the same scale.
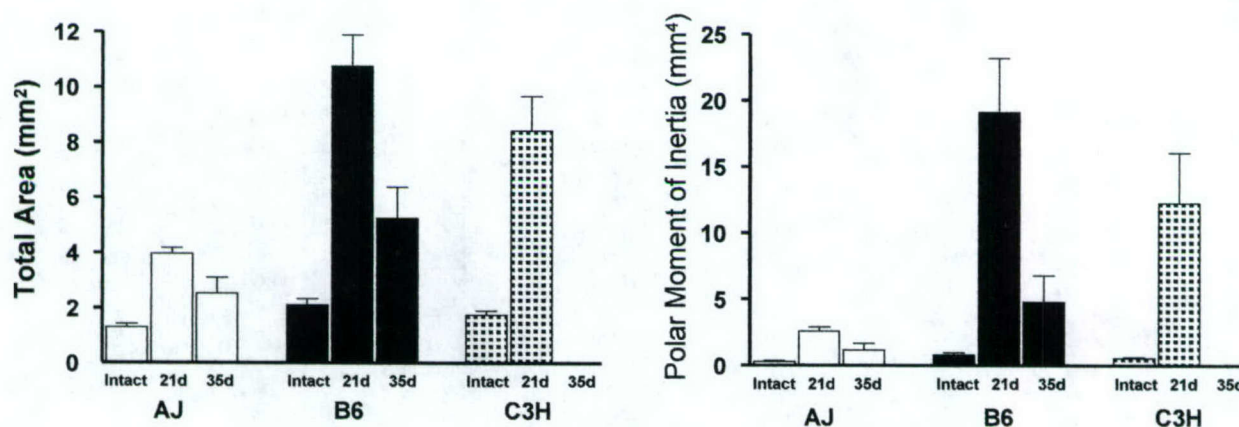

Figure 2. Variation in total bone area and the polar moment of inertia for intact femurs as well as the fracture callus.
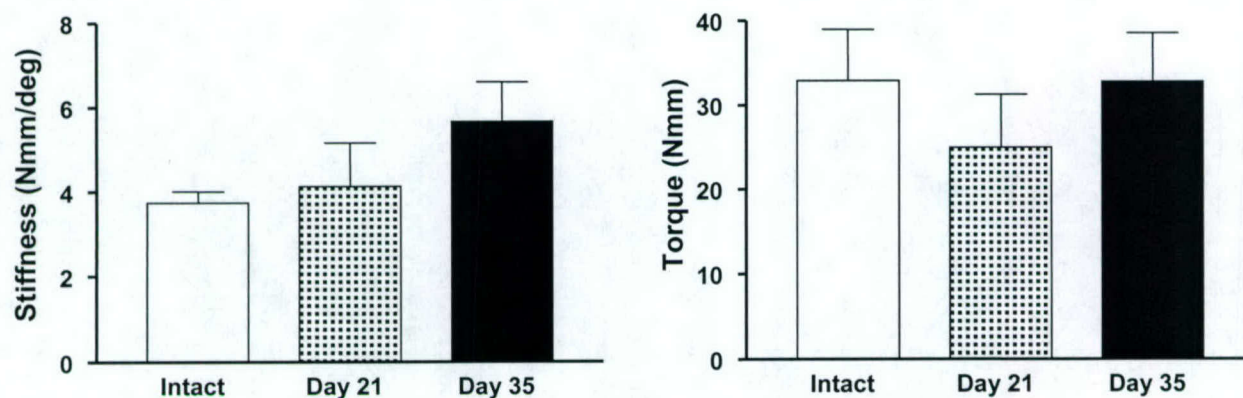
Figure 3. A/J femurs (intact and at day 21 and 35 of fracture healing) were evaluated for mechanical performance. Day 21 callus showed similar stiffness as the intact bones, however, the torque-to-failure was reduced. By day 35, the stiffness was significantly greater than the intact bone and the torque-to-failure was equivalent to that of the intact. This data indicated that A/J femurs regain strength following fracture by 35 days of age.

Reportable Outcomes

Papers
Wang K, Vishwanath P, Eichler G, Edgar C, Einhorn TA, Smith T, Gerstenfeld LC. Transcriptional profiling across fracture healing: temporal relationships between metalloproteinase and adamts messenger RNA expression during endochondral bone resorption. Journal of Cellular Biochemistry, in preparation.

Abstracts
Wang KH, Vishwanath P, Eichler G, Edgar CM, Smith T, Gerstenfeld LC. Large scale transcriptional profiling across the time course of fracture healing. Presented at the 26th Annual Meeting of the American Society for Bone and Mineral Research. Seattle, Washington, USA. October 1-5, 2004. **(POSTER)**

Wang K, Vishwanath P, Eichler G, Edgar C, Smith T, Gerstenfeld LC. Large scale transcriptional profiling across the time course of fracture healing. Presented at the 8th International Conference on the Chemistry and Biology of Mineralized Tissues. Banff, Alberta, Canada. October 17-22, 2004. **(POSTER, Young Investigator Award)**

Wang K, Vishwanath P, Eichler G, Edgar C, Einhorn TA, Smith T, Gerstenfeld LC. Transcriptional profiling across fracture healing: distinct temporal clustering of heat shock and metalloproteinase genes. Presented at the 51st Annual Meeting of the Orthopaedic Research Society. Washington, DC, USA. February 20 - 23, 2005. **(ORAL PRESENTATION)**

Training
Kevin Wang, Spring 2004, MA, Masters of Medical Sciences, Boston University School of Medicine, Thesis Title: Large Scale Transcriptional Profiling Across The Time Course of Fracture Healing.

Conclusions

1) The basic differences in bone quality as reflected in geometric biomechanical and material property within different inbred strains of mice are recapitulated during fracture healing.

2) These data indicate that the underlying genetic mechanisms that are operative during developmental bone formation reemerge during fracture healing and suggest that the processes of fracture healing may potentially be used to identify those genetic components that regulate bone quality.

3) While there are fundamental genetic differences in bone quality between the inbred strains, each strain of mouse develops its own mechanism to heal their bones in a manner that regains adequate function and creates a mechanically stable callus, but in very different ways.

4) Transcriptional profiling over fracture repair can provide both qualitative and quantitative data to identify multiple molecular mechanisms that will be essential in the fracture healing processes and potentially provide insight into those mechanisms that are variable between the various strains of mice.

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Appendices

Appendix 1.　　　Wang KH, Vishwanath P, Eichler G, Edgar CM, Smith T, Gerstenfeld LC. Large scale transcriptional profiling across the time course of fracture healing. Abstract from the 26th Annual Meeting of the American Society for Bone and Mineral Research. Seattle, Washington, USA. October 1-5, 2004.

Appendix 2.　　　Wang KH, Vishwanath P, Eichler G, Edgar CM, Smith T, Gerstenfeld LC. Large scale transcriptional profiling across the time course of fracture healing. Poster presented at the 26th Annual Meeting of the American Society for Bone and Mineral Research. Seattle, Washington, USA. October 1-5, 2004.

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Appendix 4.　　　Wang K, Vishwanath P, Eichler G, Edgar C, Einhorn TA, Smith T, Gerstenfeld LC. Transcriptional profiling across fracture healing: distinct temporal clustering of heat shock and metalloproteinase genes. Abstract from the 51st Annual Meeting of the Orthopaedic Research Society. Washington, DC, USA. February 20 - 23, 2005.

Appendix 5.　　　Wang K, Vishwanath P, Eichler G, Edgar C, Einhorn TA, Smith T, Gerstenfeld LC. Transcriptional profiling across fracture healing: temporal relationships between metalloproteinase and adamts messenger RNA expression during endochondral bone resorption. Journal of Cellular Biochemistry, in preparation.
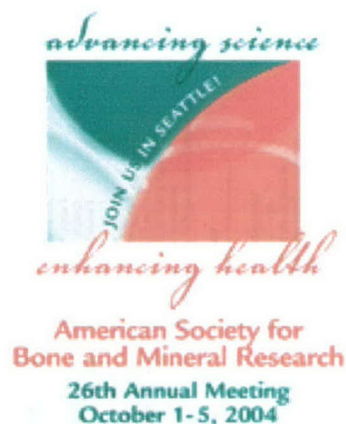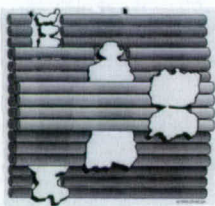
Large Scale Transcriptional Profiling Across the Time Course of Fracture Healing

*Kevin Wang[1], Prashanth Vishwanath, Gabriel Eichler[2], Cory Edgar[1], Temple Smith[2] and *Louis C. Gerstenfeld[1]
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Intorduction: Skeletal tissue regeneration occurs through the recapitulation of many aspects of the original endochondral developmental process that formed the osseous skeleton during embryogenesis. The repair process that is initiated in response to fracture entails the complex interaction of multiple cell types and cellular processes and is reflected in the complexity of the transcriptional expression that occurs during fracture healing. **Purpose:** The transcriptional expression of ~13,000 genes was assessed over the first 21 days of fracture healing. **Methods:** standard mid-diaphyseal tibia fractures were generated in C57B6 murine tibiae. The profile of transcriptional expression of ~13,000 genes was carried out across the time course of fracture healing using 75mers covalently linked onto oligo-nucleotide micro-array chips. Ribonuclease protection analysis was used for a small number of candidate genes, to verify the time course of chondrogenic and osteogenic gene expression and validate the accuracy of the predictive values of the micro array analysis. The data that were obtained from these experiments were analyzed using a novel algorithm called a Gene Expression Dynamics Inspector (GEDI) that produces self organizing maps of the expressed genes, which are graphically presented as colored coded mosaics that enables individual clusters of genes to be identified, easily visualized and associated with various temporal stages of fracture healing. **Results:** Using unfractured bone tissue as the reference and ln 2 fold greater or lesser from the reference, ~1000 mRNAs within ~13,000, were identified that varied across the time course of healing. These data identified the heat shock (HSP) gene family to be tightly expressed immediately after injury at day 3 and is the first demonstration that this family of stress related genes, which is activated in response to burn or chemical toxicity is also activated as a consequence of mechanical trauma. On the other hand a sub family of genes within the metalloproteinase (MMP) family was identified as having a complex pattern of expression during the chondrogenic and osteogenic phases of fracture healing. Showing both excluded expression during chondrogenesis MMP8 and elevated expression during endochodnral resorption (MMP2,9,13 and 14). **Conclusions:** These data demonstrate the potential uses of a global transcriptional profiling approach to identify complex networks of gene interactions that are regulated over the time course of fracture healing.

Appendix 1

8th International Conference on the
Chemistry and Biology of Mineralized Tissues

The Banff Centre, Banff, Alberta, Canada. October 17-22, 2004

Large Scale Transcriptional Profiling Across the Time Course of Fracture Healing

*Kevin Wang[1], Prashanth Vishwanath [2], Gabriel Eichler[2], Cory Edgar[1], Temple Smith[2] and *Louis C. Gerstenfeld[1]
[1] Boston University School of Medicine, [2]Boston University School of Biomedical Engineering

Intorduction: Osseous skeletal tissue is one of a few tissues in an adult vertebrate that fully regenerates in response to injury. Skeletal tissue regeneration occurs through the recapitulation of many aspects of the original endochondral developmental process that formed the osseous skeleton during embryogenesis. The repair process that is initiated in response to fracture entails the complex interaction of multiple cell types and cellular processes and is reflected in the complexity of the transcriptional expression that occur during fracture healing. **Purpose:** The transcriptional expression of ~13,000 genes was assessed over the first 21 days of fracture healing. **Methods:** standard mid-diaphyseal tibia fractures were generated in C57B6 murine tibiae. The profile of transcriptional expression of ~13,000 genes was carried out across the time course of fracture healing using 75 nt oligo-nucleotide micro-array chips. Ribonuclease protection analysis was used for a small number of candidate genes, to verify the time course of chondrogenic and osteogenic gene expression and validate the accuracy of the predictive values of the micro array analysis. The data that were obtained from these experiments were analyzed using a novel algorithm called a Gene Expression Dynamics Inspector (GEDI) that produces self organizing maps of the expressed genes, which are graphically presented as colored coded mosaics that enables individual clusters of genes to be identified, easily visualized and associated with various temporal stages of fracture healing. **Results:** Using unfractured bone tissue as the reference and ln 2 fold greater or lesser from the reference, ~1000 mRNAs within ~13,000, were identified that varied across the time course of healing. These data identified the heat shock (HSP) gene family to be tightly expressed immediately after injury at day 3 and is the first demonstration that this family of stress related genes, which is activated in response to burn or chemical toxicity is also activated as a consequence of mechanical trauma. On the other hand a sub family of genes within the metalloproteinase (MMP) family was identified as having a complex pattern of expression during the chondrogenic and osteogenic phases of fracture healing. Showing both excluded expression during chondrogenesis MMP8 and elevated expression during endochodnral resorption (MMP2,9,13 and 14) **Conclusions:** These data demonstrate the potential uses of a global transcriptional profiling approach to identify complex networks of gene interactions that are regulated over the time course of fracture healing.

TRANSCRIPTIONAL PROFILING ACROSS FRACTURE HEALING: DISTINCT TEMPORAL CLUSTERING OF HEAT SHOCK AND METALLOPROTEINASE GENES

[1]Wang, K; [2]Vishwanath, P; [2]Eichler, G; [1]Edgar, C; [1]Einhorn, TA; [2]Smith, T; [1]Gerstenfeld, LC

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INTRODUCTION

Many previous studies of fracture healing have focused on the activities of individual genes that are of functional importance during the differentiation of skeletal cell types. The biological complexity of fracture healing however suggests that it is regulated by hundreds, if not thousands, of transcriptionally activated genes involved in many different biological processes that are mediated both by skeletal and non skeletal cell types. In order to understand how the activities of such a large number of genes are coordinated, and how their activities are associated with given cellular and biological processes, recent innovations in the ability to analyze genome wide transcriptional activities can be used. Such approaches enable the transcriptional activities of large numbers of genes to be assessed and the complex interactions of genes to be organized and visualized in an understandable and interpretable manner. In this initial study we have carried out a large scale transcriptional expression profile analysis on a limited time course (3, 10 and 21 days) of murine fracture healing. We identified ~1000 expressed genes that varied log 2 or greater from unfractured bone. Further we show that using a self organizing mapping approach and post analysis multicolored two dimensional graphical mosaics that novel and specific functional groups of genes can be identified, that appear to be co regulated at different stages of fracture healing.

MATERIALS AND METHODS

Generation of Murine Tibia Fractures Research was conducted in conformity with all Federal and USDA guidelines, as well as an IACUC approved protocol. All studies were performed on male 8-10 weeks old C57BL/6J (B6) mice. A modification of the method of Bonnarens and Einhorn 198 was used to create simple transverse closed unilateral fractures in the left tibia of all mice. Fracture healing were assessed at the inflammatory phase (day 3); a mid time point in the endochondral phase (day 10); and a period of primary bone formation and coupled remodeling (day 21).

RNA Preparation Fracture calluses tissues were powdered under liquid nitrogen and total RNA was twice extracted by Trizol (Gibco BRL, Gaithersburg, MD, USA). After isopropanol precipitation RNA was dissolved in RNase free water and further purified using an RNeasy mini column (Qiagen Inc, Valencia CA) according to the manufacturer's instructions.

Transcriptional Profile Analysis: A ~13,000 feature oligonucleotide library http://pga.mgh.harvard.edu/Parabiosys/resources/ microarrays/oligonucleotides/mouse_13443_prn.txt Annotated list) was used for microarray slide construction. The basis of the oligo library is a ~70 mer oligo Operon series that is commercially available from Qiagen Inc. Slide printing, array labeling, hybridization, and slide reading were performed at the Massachusetts General Hospital Genomics Core Facility. All slides were quality control tested and contained appropriate positive and negative control sequences for data analysis. In these experiments, RNA from unfractured tibiae was used as the reference For each experiment two samples (a reference and a test) RNA were reverse transcribed into amine modified cDNA using Cy3/Cy5 labeling flouros using a modified protocol of the Atlas Power script Fluorescent Labeling Kit (Clontech Cat # K1860-1) protocol. After labeling and slide hybridization, data was collected. All oligos (genes) were ranked as a function of the intrinsic variability within the control(s). Thus any oligos that displayed high controls intrinsically suggested either that there was some problem in the oligo design or in the behavior of its associated gene. The former could often be distinguished if there were two or more oligos for the same gene on the array. Next, we calculated the replica variation for each oligo (gene) for each RNA sample hybridized. This variation was considered to be one of the attributes associated with each measured color intensity. Once the initial data had been filtered subsequent data analysis was performed.

Gene Expression Analysis: Gene Expression Dynamics Inspector (GEDI) software was used to generate self-organizing maps (SOM's), as opposed to other analysis algorithms such as hierarchical clustering (Eisen, 1998: Tamayo, 1999). High-dimensional gene expression profiles were displayed as colored coded mosaic images. Based on positional placement of a genes expression within the mosaic the patterns of gene expression may be easily identified without any prior assumptions about the genes expression (Eichler, 2003).

RESULTS

Approximately 1000 unique genes were identified that had expression at log 2.0 fold variant greater or lesser than the reference. The number of mRNAs with a log 2 fold variation from the reference unfractured bone was greatest at days 3 and 10 days post fracture while by 21 days far fewer genes showed such unique changes in their expression, demonstrating that as the fracture healed the compliment of unique genes that were turned on or off diminished as cellular compliment and activity within the healing fracture callus returned to its pre fracture makeup. Specific genes showing 3 X log 2 that are specifically induced during the endochondral period included perlecan, cartilage oligomeric matrix protein (COMP) and matrix gla protein (MGP). Other mRNAs of interest that were induced early and persisted through out the time course of healing were osteoblast specific factor 2 (fasciclin I-like) OSF-2 and osteoglycin which become elevated respectively at day three and day 10 and which remain at a high level of expression. It is interesting to note that while the MMP family is induced at very significant levels at day 10 post fracture the cathepthic enzymes and carbonic anhydrases 2 that are known to be produced by osteoclasts were significantly diminished from the reference values in native bone at this time point. Such findings therefore suggest that the recruitment of osteoclasts into the cartilaginous callus tissue is very tightly controlled and subsequent to the initiation of the proteolytic activities of the MMPs. In the studies performed here a self organizing mapping (SOMs) approach was used to identify potential groupings of genes that might be of functional significance during fracture repair. Two groups of genes that were identified in this manner were the heat shock (HSP) gene family and a subgroup of the matrix metalloproteinase (MMP) family of genes MMPs (2, 9,13 and 14) that all showed a rapid and elevated pattern of expression beginning soon after fracture and peaking during the endochondral period. Thereafter these genes expression remained at peak levels throughout the period of primary remodeling. A second group of genes that we identified using SOMs was the heat shock family of genes. This group of genes was tightly regulated immediately after fracture at day three. It is interesting to note that while the quantitative levels of expression for any of the HSP genes was less than ln 2 of our initial filtering the SOMs analysis identified this family of genes as a uniquely expressed cluster. Their expression patterns differed than those of the MMPs in that their levels of expression rapidly decreased after the first time point to levels below that of the reference.

DISCUSSION

These data demonstrate that the potential utility of assessing the complexity of transcriptional activation during fracture healing. They further showed that a group of metalloproteinase family member genes appears to concurrently up-regulated during the endochondral phase of bone healing. Finally this is the first demonstration that the heat shock family of genes may be activated in response to mechanical trauma.

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TRANSCRIPTIONAL PROFILING ACROSS FRACTURE HEALING:

TEMPORAL RELATIOSHIPS BETWEEN METALLOPROTEINASE AND ADAMTS

MESSENGER RNA EXPRESSION DURING ENDOCHONDRAL BONE RESORPTION

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Running Head: Transcriptional Profiling In Fracture Healing

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ABSTRACT

The repair process that is initiated in response to fracture entails the complex interaction of multiple cell types and biological processes, and is reflected in the complexity of the transcriptional expression that occurs during fracture healing. Standard mid-diaphyseal tibia fractures were generated in C57B6 murine tibiae and the transcriptional expression of ~13,000 genes was assessed using 70 nt oligo-nucleotide microarray slides. Using unfractured bone as the reference the transcriptional activity of genes with 2 fold greater or lesser levels of expression relative to the reference was identified. Using a self-organizing mapping approach, the temporal relationships between extracellular matrix protein expression and that of the proteases that degrade proteoglycan and collagenous matrices was assessed. A very broad group of extracellualr matrix mRNAs representative of connective tissues in general and more specialized proteins expressed predominantly in basement membranes, blood vessels and in cartilage all showed an elevated pattern of expression over a 21 day period after fracture and their expression peaked at 10 days when the endochondral period of the bone formation was maximal. Specific metalloproteinase mRNAs were consecutively expressed with MMP 14 and 2 preceding MMP9 and MMP13 while the expression of MMPs 8 and 19 showed a strong inhibition of their expression during the first 21 days of bone healing. The metalloproteinase inhibitors (TIMPs 2 and 3 had an identical pattern of expression as MMPs2 and 14 while TIMP1 was induced early after fracture and remained highly expressed thereafter. The disintegrins/ADAMTS family of proteases also showed very unique patterns of expression with Adamts 4 and 15 paralleling the expression of MMPs2 and 14 and ADAMTS 1 following the expression pattern of MMPs 9 and 13. A second group (ADAMTS 17,19,5 and 8) showed a biphasic

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pattern of expression first being induced right after fracture then showing reduced

expression during the endochondral period followed by a period of increasing expression

during primary bone remodeling. These results demonstrate that there is an orderly

temporal expression of MMPs and ADAMTs that may be necessary for the progression of

endochondral resorption. Furthermore based on their patterns of expression, relative to

the known activities of the encoded proteolytic enzymes, our results suggest that the

dissolution of cartilage protoeglycans proceeds before the underlying collagenous

components of the matrix are removed.

INTRODUCTION

While the traditional one gene one function paradigm has been the basis of contemporary biological understanding for almost a century, it is increasingly becoming clear that in multicellular organisms, many developmental and homeostatic physiologic processes are regulated by a series of complex, temporally changing, and redundant patterns of multiple expressed genes. Osseous skeletal tissues are one of the few tissues in adult vertebrate animals that regenerate in response to injury. During fracture healing, a repair process is initiated that entails the complex interaction of multiple cell types and many different biological processes (Ferguson et al., 1999; Gerstenfeld et al., 2003; Vortkamp et al., 1998). Thus fracture healing is a unique model to identify the relationships between the mechanisms that regulate specific responses to a traumatic injury and the genetic components that control the development of bone. The identification and understanding of interactions of the various genomic components that both respond to injury, affect bone development and regulate repair are therefore of considerable clinical importance. Identifying these molecular mechanisms may help us develop genetically based predictive indices to assess the healing potential of various types of fractures as well potential therapeutic interventions to promote healing.

Many previous studies of fracture healing have focused on the activities of individual genes that are of functional importance during the differentiation of skeletal cell types. The biological complexity of fracture healing however suggests that it is regulated by hundreds, if not thousands, of transcriptionally regulated genes involved in many different biological processes that are mediated both by skeletal and non skeletal cell types. In order to understand how the activities of such a large number of genes are coordinated, and how their activities are associated with given cellular and biological processes, recent innovations in the ability to analyze genome

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wide transcriptional activities can be used (Hadjiargyrou et al., 2002; Desai et al., 2003). Such approaches enable the transcriptional activities of large numbers of genes to be assessed and the complex interactions of genes to be organized and visualized in an understandable and interpretable manner.

In this initial study we have carried out a large scale transcriptional expression profile analysis on a limited time course (3, 10 and 21 days) of murine fracture healing. We identified several hundred expressed genes that were over 2 fold differentially expressed compared to unfractured bones. In this first study, we focused our analysis on the nature of the compliment of extracellular matrix proteins that were expressed during fracture healing and two specific proteolytic enzymes families, the metalloproteinases and ADAMTS, that are largely responsible for remodeling extracellular matrices. We used a self-organizing map (SOM) (Kohonen et al., 2002; Eicher et al., 2003; Tamayo et al., 1999) based approach to aid in the visualization and cluster analysis of the time series data which identified specific functional groups of these genes that appear to be co-regulated at different stages of fracture healing.

MATERIAL AND METHODS

Generation of Murine Tibia Fractures Research was conducted in conformity with all Federal and USDA guidelines, as well as an IACUC approved protocol. All studies were performed on male 8-10 weeks old C57BL/6J (B6) mice. Age, sex and genetic strain of mouse were based on previous data, in which the time course of fracture healing had been established by both histological and selected candidate gene assessment (Kon et al., 2001; Cho et al., 2002; Gerstenfeld et al., 2003). A modification of the method of Bonnarens and Einhorn (1984), as previously described (Kon et al., 2001) was used to create simple transverse closed unilateral fractures in the left tibia of all mice. Fracture stabilization by intramedullary fixation was carried

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out using the stylet of a 25G spinal needle (Becton Dickinson, Franklin Lakes, NJ, USA). For enrollment in our studies, the location and quality of fractures were assessed immediately after the fractures had been generated by x-ray analysis. A fracture, whose configuration was inconsistent with standardized placement criteria (mid-diaphyseal) or observed to be grossly communited were not used in our studies. Mice were euthanized at selected time points after fracture by CO_2 asphyxiation

Time points of analysis: Specific time points over the course of fracture healing were chosen to assess representative periods in the inflammatory phase (day 3); a mid time point in the endochondral phase (day 10); and a period of primary bone formation and coupled remodeling (day 21). Selection of these points was based on empirical knowledge of this model.

RNA Preparation After euthanasia limbs were rapidly disarticulated and surrounding muscular was carefully removed with care being taken to not disrupt the fracture site or callus tissues. Fracture sites were circumscribed by 5 mm on either side of the break site and an identical site was excised from the mid half of the unfractured contralateral tibiae. The fracture calluses were collected into liquid nitrogen and stored at -80 °C until ready for RNA extraction. Tissues were powdered under liquid nitrogen and total RNA was extracted by Trizol (Gibco BRL, Gaithersburg, MD, USA) as previously described (Kon et al., 2001). RNAs to be used for microarray were redissolved in Trizol and extracted a second time. After isopropanol precipitation RNA was dissolved in RNase free water and further purified using an RNeasy mini column (Qiagen Inc, Valencia, CA) according to the manufacturer's instructions. At this point, the RNAs are precipitated with Na Acetate 300mM and 2.5 volumes of 100% EtOH and stored until use.

Ribonuclease Protection Assay Analysis: For validation of individual genes ribonuclease protection assays (RPA) were carried out. All template sets were from Pharmingen Corp. Assays for bone extracellular matrix genes were carried out with 3μg of total mRNA and 10μg was used for the assay of the MMPs. Single stranded 32P-labeled cRNA probes and denaturing acrylamide gels analysis were as previously described (Cho et al., 2002). All data sets were normalized to the housekeeping gene L32.

Analysis of Genomic Data Labeled samples were hybridized to slides containing the Operon Mus musculus V1.1 probe set (Qiagen) of over 13,000 70-mer oligonucleotide probes printed by the Massachusetts General Hospital Microarray Core Facility. Slide printing, array labeling, hybridization, and slide reading were performed at the Massachusetts General Hospital Genomics Core Facility. All slides were quality control tested and contained appropriate positive and negative control sequences for data analysis. In these experiments, two different reference RNAs were used. The initial trial used an RNA reference made from a mixture from 12.5 and 17 day p.c. whole mouse embryos. Two embryos were used for each time point and separate time point samples were mixed together to produce the reference RNA. In the second trial, RNA from unfractured tibiae was used. For each experiment two samples (a reference and a test) of RNA were reverse transcribed into amine modified cDNA using Cy3/Cy5 labeling flouros using a modified protocol of the Atlas Power script Fluorescent Labeling Kit (Clontech Cat # K1860-1) protocol. After labeling and slide hybridization, data was collected. A GenePix 4000B microarray scanner and software (Axon Instruments) were used to quantify the microarrays, and data were analyzed using the BioArray Software Environment (BASE) Quality and Array Standardization: Once the hybridization profiles were collected from the experiments, initial improvements for the use of internal or comparative controls for the

experiments were performed. This was done by at least two different methods. Either multiple

preparations of the common RNA control sets were prepared or the same sample was run

through any sequence or time course through which it was anticipated that all other samples were

to be put. Each of these was then compared in a two-color reciprocal manner. In this manner all

oligos (genes) were ranked as a function of the intrinsic variability within the control(s). Thus

any oligos that displayed high controls intrinsically suggested either that there was some problem

either in the oligo design or in the behavior of its associated gene. The former could often be

distinguished if there were two or more oligos for the same gene on the array. Next, we

calculated the replica variation for each oligo (gene) for each RNA sample hybridized.

Data normalization and Filtering The data was further normalized by scaling all

individual intensities such that mean total intensity was the same for both comparative samples

(control and stimulated) within a single array and across replicates. The replicates were then

combined to reduce the complexity of the data set. Genes with only one data point after initial

selection were excluded. Genes with more than one replicate data point were then analyzed for

outliers and discarded if necessary. The data was combined using the geometric mean of the

replicate ratios. Non-uniform features and genes with low intensities not significantly above

background were excluded from further analyses.

Using the standard $\log_2$ ratio of the two dye intensities from each array spot representing

each gene, the distribution of the log ratios was obtained for both the individual and combined

replicates. In general these distributions are sufficiently normal so that a mean ratio and standard

deviations could be calculated. This allows one to identify genes that were expressed

sufficiently above the noise without having to resort to an arbitrary minimum ratio value. The

data was then visualized using log ratio-intensity versus log intensity product on a standard R-I

plot, see Figure 2 The plot reveals the intensity-specific artifacts in the $\log_2$ ratio measurements. The one and two standard deviation cutoffs also reveal the inherent "noise level" limitation in the $\log_2$ ratio measurements. We carried out a number of analyses for those genes for which data was available across all time points. The first step was to concatenate the $\log_2$ expression ratios of all four time points to form one set of vectors. Genes that did not change by more than two standard deviations (as defined by the RI plot) in at least one time point were discarded to reduce complexity in later analysis.

Gene Expression Dynamics Inspector (GEDI) Once a final dataset of high quality probe measurements was attained, we employed the Gene Expression Dynamics Inspector (GEDI) software. This software package uses self-organizing maps (SOM's), as opposed to other analysis algorithms such as hierarchical clustering (Eisen et al., 1998; Tamayo, 1999) and displays high-dimensional gene expression profiles as colored coded mosaic images. Based on positional placement of a genes expression within the mosaic the patterns of gene expression may be easily identified without any prior assumptions about the gene's expression (Eichler et al., 2003). Utilization of this software enabled us to analyze both global dynamics of several thousand genes as well as to investigate the specific patterns of expression of individual genes.

RESULTS

Because of the potential variability in the progression of fracture repair within any one animal, the expression profile of a cassette of extracellular matrix protein genes was initially assessed in each set of RNA replicates to ensure that it was representative of the normal temporal pattern of endochondral bone formation (Figure 1). The inflammatory phase of fracture healing (day 3), was characterized by an initial decrease in the expression of the major mRNA markers of differentiated osteogenic cells (osteocalcin type I collagen and bone sialoprotein) (Kon et al.,

2001). This is due both to the large influx of non osteogenic cells within the hematoma that forms in response to the fracture. At this time large numbers of skeletogenic precursors that will eventually contribute to the initial repair tissue that forms have been recruited but do not yet express skeletal tissue phenotypes. Consistent with this interpretation was the increased expression of osteopontin which is both expressed by macrophages and other inflammatory cells (Kon et al., 2001) as well as early skeletalgenic precursors (Barnes et al., 1999; Bolander, 1992). Day 10 is representative of the peak of the endochondral phase and is characterized by the appearance of high levels of both collagen types II and X validating the peak level of cartilage tissue formation that accompanies endochondral bone formation. It is interesting to note the lower levels of osteopontin during this period and higher levels of bone sialoprotein which are seen both during cartilage hypertrophy and as a marker of early to mid osteogenic differentiation (Barnes et al., 2002). Day 21 is representative both of primary bone formation and coupled remodeling. At this time point, the profile of expressed mRNAs has almost returned to that seen in unfractured bone but the levels of osteocalcin remain elevated above the baseline of normal bone.

While the RPA analysis is an important assessment of RNA integrity and provides a means of confirming the temporal progression of the repair process, microarray experiments necessitate a much higher purity which can only be emperically determined by carrying out an actual microarray experiment. A representative area from one of the microarray slides for a typical experiment is presented in Figure 2. As can be seen excellent spot development and very low levels of background were observed demonstrating that the RNA extraction and purification procedures that were developed for these skeletal tissues produced the high degree of purity of the RNA samples needed for these studies. The raw data for the spot intensity of osteocalcin

expression, a know gene to be highly restricted to bone are presented. In order to validate the integrity of the profiling experiments, the read out of the spot intensity relative to the observed expression profiles seen with RPA can be compared. As can be seen the spot development in both channels showed an excellent correspondence to the observed hybridization profiles from the RPA assay. In the initial trial of this study pooled mRNAs from 12.5 pc and 17 pc day whole mouse embryos had been used as the reference and in these experiments this mRNA shows near absence of expression during embryogenesis with in the mouse, consistent with previous published data. In this context it is also interesting to note that the duplicated form of this gene the "osteocalcin related gene" showed an identical pattern of expression as osteocalcin (data not shown) (Desbois et al., 1994).

Overall in the first trial approximately about ~1000 unique genes were identified that had unique expression at 3 fold variant from the reference and this first trial validated the integrity of the slides and the RNA replicates. We found however there was an inherent bias in detecting mRNA expression that emerges during fracture healing because many of these same mRNAs are expressed during embryogenesis. In our second experiment unfractured bone mRNA was used as the reference. Transcripts that are over 3 fold differentially expressed as compared to the control samples were identified. This data set, which presented is presented here is based on a minimum of three replicate assays from two pools of RNA. Table 1 and Figure 3 summarizes this data set. A comparison of the graphic presentation of these data in figure 3 shows that the number of observed mRNAs at each time with a 3 fold variation from the observed levels in reference unfractured bone was greatest at days 3 and 10 days post fracture while by 21 days far fewer genes showed such unique changes in their expression. This data demonstrates that as the fracture healed the compliment of genes that were differentially expressed from those expressed

in bone during normal homeostasis diminished as cellular compliment and activity within the healing fracture callus returned to its pre fracture makeup (Figure 3). Thus the complexity of the number of biological processes that are concurrently operative during this early period of fracture healing is much greater than that which occurs during the later period of primary and secondary bone formation.

A number of specific genes worth noting are highlighted in Tables 1. In particular genes that are specifically induced at very large degrees of variance from the reference during the endochondral period included, perlecan, cartilage oligomeric matrix protein (COMP) and matrix gla protein (MGP) all three of which have been known to be highly expressed in developing cartilage but not in normal bone. Other mRNAs of interest that were induced early and persisted through out the time course of healing include osteoblast specific factor 2 (fasciclin I-like) OSF-2 and osteoglycin. Several specific matrix metalloproteinases and their selective inhibitors (TIMPs) were also induced selectively during the endochondral period and persisted during the period of primary bone remodeling. In references to the expression of OSF-2, osteoglycin and the TIMPs, these genes have all been identified in two separate large scale in vitro transcriptional profiling studies with various mesenchymal stem cell lines that were focused on identifying the patterns of genes that are regulated by BMPs (Balint et al., 2003; Stock et al., 2004). Finally one other mRNA that was induced throughout fracture healing was TNF super family member 22. This observation is noteworthy on two points: the product of this mRNA encodes for a TRAIL decoy receptor that can also block the activities of RANK-L and it followed the same expression pattern as the osteoprotegrin mRNA (data not shown).

It is interesting to note that while a number of matrix metalloproteinases (MMPs) are induced at very significant levels at day 10 post fracture several cathepthic enzymes as well as

carbonic anhydrases 2 that are known to be expressed at very high levels by osteoclasts

(Rousselle and Heymann, 2002) were significantly diminished from their reference values in

native bone. These findings suggest that the recruitment of osteoclasts into the cartilaginous

callus tissue is very tightly controlled and this observation is consistent with the proposal that

osteoclast activity begins only after the initiation of the proteolytic activities of the MMPs

(Blavier and Delaisse, 1995). Finally as observed both by RPA and in the first microarray

experiments osteocalcin is significantly underexpressed throughout the time course until primary

bone formation has been induced and is well underway at day 21.

While the first stage of the analysis only identified potential candidate genes of interest

based on quantitative changes in their level of expression from a reference (in this case

unfractured bone), complex patterns of functional groupings of genes that may be associated

with a given cell type or specific biological process can be identified. Changes up or down of

functional groups of genes that are expressed in discernable patterns, in relationship to the time

course of fracture healing can also be identified using any number of different types of analytical

approaches. In the studies performed here a self-organizing mapping (SOMs) approach was

used to define the temporal relationships between extracellular matrix protein expression and that

of the proteases that degrade proteoglycan and collagenous matrices during fracture healing. A

two dimensional graphic presentation of the quantitative and temporal patterns of mRNA

expression as determined from the SOMs analysis is presented in Figure 4. Table 2 presents two

groupings of extracellular matrix protein mRNAs that showed slightly different temporal

patterns of expression but which are all tightly grouped together in a small area within the

mosaic pattern. Group I shows an increasing level of expression between 3 and 14 days after

which their levels began to fall but remained above the reference. This group of mRNAs

included a large selection of genes that encode several different collagen types and extracellular cellular matrix proteins that are broadly associated with many different types of connective tissues, a second set of mRNAs that are more selectively associated with basement membranes and vascular elements, while a third set of mRNAS that are primarily restricted to cartilage specific connective tissues. The Group II genes all showed an increasing level of expression across the entire time course and included both mRNAs for type I collagen.

Since the resorption of cartilage tissue represents a major transitional phase in the processes of endochondral bone formation, two families of genes that were of considerable interest to focus on in these studies were a disintegrin and metalloproteinases domain with thrombospondin type -1 modules (ADAMTs) gene family of which a subgroup includes the aggrecanases that are responsible for the degradation of large proteoglycan found in cartilage, and the matrix metalloproteinase (MMP) genes and their specific inhibitors (TIMPs) that play roles both in mediating vascularization and collagen turnover (Figure 4-6). MMP 2 and 14, 9, 13 and TIMPs 1, 2 and 3 all showed elevated expression during the 21 days of fracture healing. Looking more specifically at the SOMs data showed that individual groups of metalloproteinase mRNAs were consecutively expressed with MMP 14 and 2 preceding MMP9 and MMP13 while the expression of MMPs 8 and 19 showed a strong inhibition of their expression during the first 21 days of bone healing. The metalloproteinase inhibitors (TIMPs 2 and 3 had an identical pattern of expression as MMPs 2 and 14 while TIMP1 was induced early after fracture and remained highly expressed thereafter.

Specific members of the ADAMTS proteases gene family also were expressed across the time course of fracture and showed very unique temporal patterns of expression. ADAMTS 4 and 15, which both have aggrecanases activity (Apte, 2004) paralleled the expression of MMPs 2

and 14. On the other hand ADAMTS1 which also has potential aggrencanase activity followed

the pattern of expression of MMPs 9 and 13. A second group (ADAMTS 17,19,5 and 8)

showed a biphasic patterns of expression first being induced right after fracture then showing

reduced expression during the endochondral period followed by a period of increasing

expression during primary bone remodeling. The final ADAMTS that we observed to be

expressed in these tissues was ADAMTS 10 which followed the pattern of MMP8 and 19

DISCUSSION

Studies of developmental, pathological, and pharmacological mechanisms are

progressively making use of transcriptional profiling and computational analysis to shed

understanding on these processes (Su et al., 2002). The use of large scale transcriptional profile

analyses for disease diagnostics has also been well documented over the last several years

(Golub et al., 1999; Armstrong et al., 2002). Indeed, a preliminary report of functional mRNA

expression during fracture healing suggested that the Wnt signaling system is uniquely activated

at defined times during rat fracture healing (Hadjiargyrou et al., 2002). It is therefore interesting

to note that independent concurrent human kindred analysis of an autosomal dominant

syndrome characterized by high bone mineral density identified a mutation in the LDL-receptor

related protein –5 that was mechanistically shown to correlate to aberrant Wnt signaling activity

within the affected kindred (Boyden et al., 2002). A comparison of these two studies, then

demonstrates that both candidate gene and transcriptional profiling approaches have equal

potential in the discovery of underlying mechanisms that are relatable to specific biological

processes.

Large scale transcriptional profiling has also been carried out to study rat femur fracture

healing in adult and juvenile rats. Differential rates of radiographic union were observed for

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young rats that reached radiographic union by 4 weeks post injury, in contrasts to the older

animals that showed union by 8-10 weeks post injury. A comparison of transcriptional profiles

of the two ages of rat healing demonstrated quantitative variations in the expression for a number

of bone matrix related genes including osteocalcin and collagen type I and showed delayed

patterns of their maximal expression associated with aging. In this study however, many of the

expressed regulatory genes that control the initial healing process such as BMPs and those for

the extracellular matrix genes associated with endochondral development, were unaffected

leading these authors to conclude that the delayed union in older animals was not due to an

altered repair process during endochondral bone formation but rather were related to other genes

associated with the secondary bone formation (Desai et al., 2003).

While the study by Desai et al. (2003) was the closest in technical approach to our study

in that an unselected genome wide assessment of fracture healing was used, unlike our study

complex analytic methods of data analysis were not presented rather they simply reported on a

series of potential candidate gene products that showed quantitative changes across the time

course of fracture healing. In contrast both our study and that of Hadjiargyrou et al. (2002) used

cluster analysis methods to identify groupings of expressed genes that might be of potential

functional importance during fracture healing. In the studies of Hadjiargyrou et al. (2002) the

approach selected against most of the preexistent mRNAs in bone tissue by using suppressive

subtractive hybridization cloning to generate cDNAs of the RNAs that were only selectively

upregulated in fracture calluses during the time course of healing. Thus, many quantitative

positive and negative changes in the pattern of the full compliment of genes that are expressed

during facture healing were not assessed in this study.

Although a self organizing mapping approach was used in our studies, the data was visualized as 2-dimensional colored mosaics. These high-resolution mosaic pictures (Figure 4) were created from miniclusters of 1-9 genes per cluster with the resolution of these pictures being dependent on the number of SOM clusters that were evaluated. To determine the color of each tile, we looked at the centroid value of their respective minicluster in this manner the SOM organized similar genes that show similar temporal and quantitative differences are depicted in the same neighborhood of the mosaics and clear coherent images are generated of the expression profiles that are characteristic of each sample (Eichler et al., 2003). Furthermore, because our expression profiles are from multiple time points the mosaic images can be stacked into animations that provided very clear visualization of the evolution of the expression of a given cluster of expressed genes over the time course. In the end, the GEDI software gave us a means of investigating simultaneous gene activities across a time course without prior assumption of any structure in the data. This allowed us to efficiently see patterns of expression, identify genes and groups of genes of interest, and ultimately retrieve these genes to determine their role in fracture healing.

In these initial studies we focused on defining the temporal relationships between the expression profiles of extracellular matrix protein and the proteases that are involved in the remodeling and turnover of the extracellular. We observed a broad and diverse group of ECM protein genes that showed an induction in their expression during fracture healing. The expression of some of these genes such as collagen types III, IV, VI ,XVI , and laminin while having all been examined during normal bone and endochondral development (Durr et al., 1996; Hausler et al., 2002; Kassner et al., 2003; Luther et al. 2003), have not been reported in fracture healing or bone repair and may merit further examination.

Two biological processes of fracture healing that appear to be critical to the progression of the endochondral bone formation and bone repair are tissue vascularization and the resorption of the endochondral tissues (Vu et al. 1997 ; Street et al., 2002; Gerber et al., 1999). Previous studies have shown that MMPs carry out essential roles during both normal skeletal development and during adult fracture repair and are uniquely activated during endochondral development (Colnot et al., 2003; Holmbeck et al., 2003; Ortega et al., 2003; Zhou et al., 2000; Jimenez et al., 2001). A number of other studies have also shown that many of the MMPs are activated during the pathological turnover of cartilage during both rheumatoid and osteo arthritic processes (Cho et al., 2003; Glasson et al., 2004). While many studies have focused on individual MMPs only a few have tried to assess how the proteolytic activities of the various MMPs are coordinated. Our studies would suggest that the expression TIMP2/MMP2/ MMP14 and MMP9/MMP13 are two separate groups that coordinately regulated. In this context several recent studies have shown that MMP14/MT-1, MMP2 and TIMP2 are associated together in a protein complex and that pro MMP2 is only activated in conjunction with MMP14 and TIMP2 (Apte et al 1997, Jo et al., 2000; Karagiannis et al., 2004).   Studies of both pathological turnover of articular cartilage, as well as normal developmental remodeling of endochondral cartilage, have shown that MMP9 and MMP13 are also concurrently expressed (Vu et al 1998, Lehman et al., 2004; Kevorkian et al., 2004). One final aspect about these data that is worth discussing, relates to the lack of expression of the MMP 8 during the endochondral phases of fracture healing. Since MMP8 is found predominantly in nuetrophils, these data would suggest that this cell type is largely absent in the healing tissues, until the marrow element has been reestablished during the late phases of primary bone formation. MMP8 mRNA has also been shown to be expressed at very levels during the pathological turnover of cartilage associated with osteoarthritis but is found at higher

levels in rheumatoid arthritis suggesting that osteoarthritis has some similarities to a normal endochondral process (Glasson et al., 2004).

Much less is know concerning the expression and mechanisms of remodeling and resorption of cartilage proteoglycans during endochondral development, although it is now clear that members of the ADAMTS family of proteases appear to be present in cartilage (Kevorkian et al., 2004; Tortorella et al., 1999; Apte, 2004) While several studies have shown that that ADAMTS 4 and 5 are in abundance in both in osteoarthritic cartilage and during endochondral remodeling, transgenic animals with deficiencies in ADAMTS 4 the most prevalent of aggrecanase in cartilage, still appears to undergo both normal development and progressive osteoarthritic degradation under experimental conditions (Glasson et al., 2004; Cal et al., 2002). The studies reported here are the first ones to date that have examined the expression of the ADAMTS gene family during fracture healing and showed like other developmental studies, that ADAMTS 4 is present during endochondral resorption. A second family member ADAMTS 15, followed the expression of ADAMTS 4. ADAMT 15 based on sequence analysis would also be considered an aggrecanases (Apte 2004) although its functional role in endochondral resorption is unproven. Two other mRNAs having aggrecanase like sequences ADAMTS 1 and 5 had different profiles from that of ADAMTS 4 and 15, with ADAMTS1 reaching maximal levels of expression slightly later in the time course while ADAMTS 5 showing a biphasic expression pattern most like the other three other ADAMTS mRNAs 8,17, and 19 that were expressed during fracture healing.

While our initial experiments were very limited relative to the number of time points and the families of gene expression that were examined, our data did create a baseline of information that demonstrates the efficacy of using transcriptional profiling techniques to assess the complex

biological processes of fracture healing. Large-scale transcriptional profiling data will be able to uncover numerous complex relationships between various molecular processes that are occurring during fracture healing when additional time points are included and fracture healing is examined in transgenic animal models.

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Figure Legends

Figure 1. RNA expression analysis of selected extracellualr matrix protein mRNAs across the time course of fracture healing by RPA. **Panel A.** Representative autoradiographic images of the RPA products as resolved on a 6% PAGE sequencing gel. Days after fracture and positions of each protected species are denoted. **Panel B.** The graphic analysis of the relative mRNA levels of selected genes. Radioactive counts of the protected bands were determined by direct β–counting on a flat surface beta detector. Band counts were normalized to the ratio of the internal standard, L32, and expressed as a relative value.

Figure 2. Representative section of a microarray slide. **Panel A.** Representative picture of a section of the hybridization slide that was double hybridized with labeled embryonic reference cDNA against fracture callus cDNA. **Panel B.** A representative example of single spot analysis for osteocalcin expression with reciprocal dye switching to demonstrating ostoecalcin expression levels as a function of time after fracture relative to a reference of a pooled sample of embryonic bone mRNA.

Figure 3. Intensity plots of total expressed mRNAs at various times after fracture. RI plots were generated by GEDI giving a visual representation of uniquely overexpressed and underexpressed genes and groups of genes. We used unfractured bone (day 0) as our reference and saw the greatest variance in uniquely expressed genes showing log 2 fold greater or lesser from the reference during the first ten days after injury. 2 standard deviations from the reference is shown with dotted lines and 1 standard deviation from the reference is shown with dashed lines.

Figure 4. Temporal Expression of MMPs and ADAMTS expression as characterized by SOMs Analysis During Facture Healing . **Panel A.** Mosaic images created by GEDI analysis of SOMs data are presented. White box is the expression position in the mosaics of the SOMs groupings of the ECM mRNAs described in table 2. Positions of the various ADAMTS are denoted by circles while the positions of the various MMPs are denoted by stars. **Panel B.** Line graphs of expression patterns of the various MMPs and ADAMTs.

TABLE 1 Selective Expression Data From MicroArray Data
Numeric data is the Log 2 value of expression relative to the reference (Unfractured bone)

Log 2	Over Expressed	Log 2	Under Expressed

DAY 3

3.73	*tissue inhibitor of metalloproteinase 1 Timp*	-2.16	*bone gamma-carboxyglutamate protein, Osteocalin*
3.7	*thrombospondin 4 Thbs4 calcium ion binding*	-2.18	acid phosphatase 5, tartrate resistant Acp5 hydrolase
3.35	fatty acid binding protein 5, Fabp5	-3.23	carbonic anhydrase 2 Car2 lyase
2.72	*osteoblast specific factor 2 (fasciclin I-like) OSF-2*	-4.16	cathepsin E ; neutrophil collagenase
		-4.44	carbonic anhydrase 2 Car2 lyase

DAY 10

4.17	*cartilage oligomeric matrix protein (Comp)*	-3.03	neutrophilic granule protein Ngp
4.05	*osteoglycin Ogn*	-3.06	*matrix metalloproteinase 8 Mmp8*
3.82	*matrix gamma-carboxyglutamate (gla) protein*	-4.48	carbonic anhydrase 2 Car2 lyase
3.81	thrombospondin 4 Thbs4 calcium ion binding	-4.93	cathepsin E ,neutrophil collagenase
3.7	follistatin-like Fstl heparin binding;	-5.69	carbonic anhydrase 2 Car2 lyase
2.98	*perlecan (heparan sulfate proteoglycan 2) Hspg2*		
2.79	midkine Mdk growth factor; heparin binding		
2.76	*tissue inhibitor of metalloproteinase 1 Timp 1*		
2.67	*matrix metalloproteinase 14*		
2.65	insulin-like growth factor binding protein 5 Igfbp5		

DAY 21

2.34	thrombospondin 4 Thbs4 calcium ion binding-	-2.62	neutrophilic granule protein Ngp
2.26	twist gene homolog Twist DNA binding; protein	-3.18	eosinophil-associated ribonuclease 3 Ear3
2.26	*tissue inhibitor of metalloproteinase 1 Timp*		
2.25	*osteoglycin Ogn*		
2.1	*matrix metalloproteinase 2 Mmp2*		

TABLE 2 Prevalent SOMS Groupings Showing Elevated Expression Throughout The Repair Period

A. Group I

Generalized ECM Proteins
Procollagen type V alpha 1
Procollagen type V alpha 3
Procollagen type VI alpha 2
Procollagen type VI alpha 1
Procollagen type VI, alpha 3
Procollagen type XI, alpha 1
Procollagen type XVI, alpha 11
Procollagen-C endopeptidase enhancer
Collagen triple helix containing 1
Procollagen-Lysine 2-Oxoglutarate 5-Dioxygenase
Osteoglycin

Muscle Related
Actinin
Desmin
Calsequestrin 1
Troponin T3
Troponin C
Tropomodulin 4
Sarcoglycan
Troponin 1
Dystroglycan
Carbonic Anhydrase 3

TNF Family
TNF receptor superfamily member 22   (TRAIL Decoy)
TNF receptor superfamily, member 11b
(osteoprotegerin)

Vessel and Basement Membrane Related
Procollagen type IV alpha 1
Procollagen type IV, alpha 2
Thrombospondin 4
Thrombospondin 2
Thrombospondin 1
Laminin beta 2
Laminin gamma 1
Lamin A
Nidogen 1
Procollagen type III alpha1
Fibrillin
Ecm1 : Extracellular matrix protein 1

Cartilage Associated
Cartilage oligomeric matrix protein
Perlecan
procollagen gene   (IX) alpha2
procollagen type   (IX) alpha 1
Ecm1 : Extracellular matrix protein

Non Defined Families
Osteoblast specific factor 2
S100 Calcium binding protein
Secretogranin II
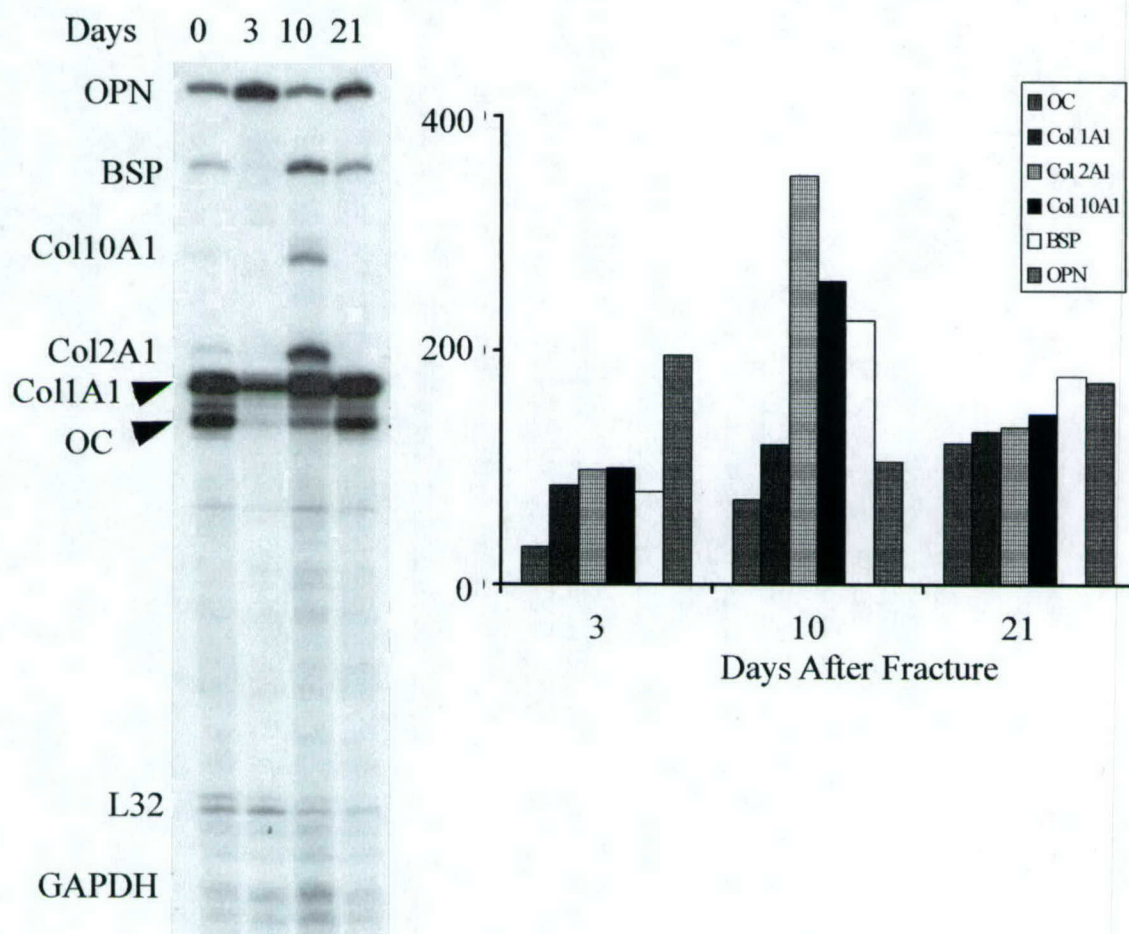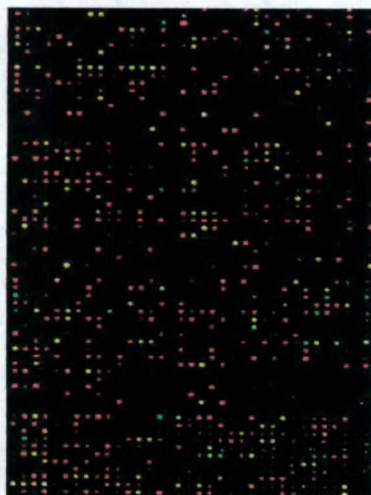Cadherin 4
Vimentin
Annexin A6

B. Group II
Procollagen type 1 alpha 1
Procollagen type I, alpha 2
Procollagen, type V, alpha 2
Procollagen C-proteinase enhancer protein

A.

FIGURE 1.

A.

B.

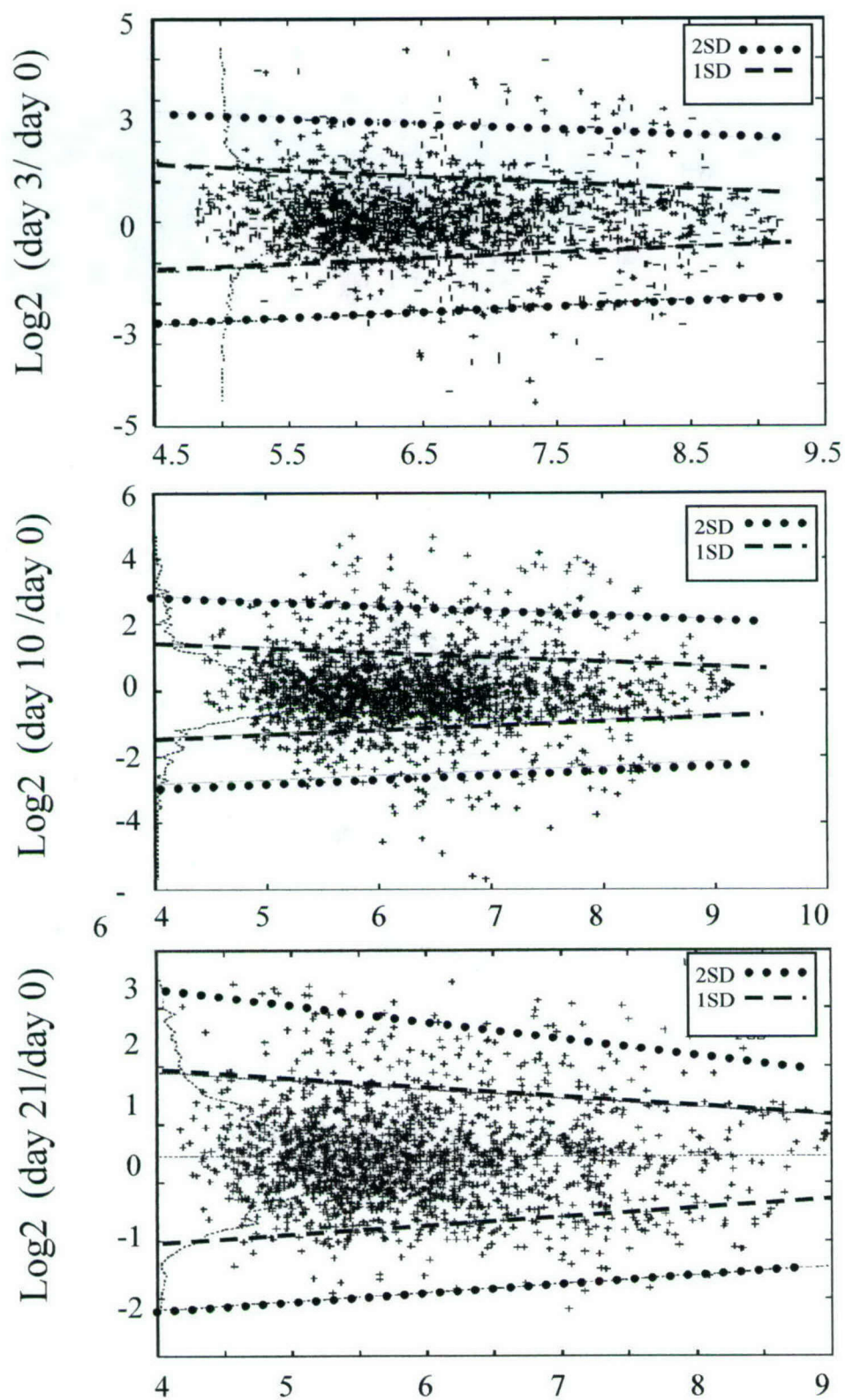| Assay	Ch1 int	Ch2 int	Ratio	log2	Spot size	Rgn. ratio	Flags	M-value	CV	Spots	Pos
Day 0	401	23409	0.02	-5.87	120	0.019	0	0	0		6472
Day 3	597	11177	0.05	-4.23	110	0.051	0	0	0		6472
Day 10	1276	63133	0.02	-5.63	100	0.022	0	0	0		6472
Day 21	34136	350	97.53	6.61	100	0.01	0	0	0		6472
Day 0	553	41015	0.01	-6.21	110	67.763	0	0	0		6472
Day 3	884	23480	0.04	-4.73	110	26.542	0	0	0		6472
Day 10	1840	64884	0.03	-5.14	100	34.334	0	0	0		6472
Day 21	64590	447	144.50	7.17	110	126.11	0	0	0		6472

FIGURE 2.

FIGURE 3.

FIGURE 4B (con't).